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# Dicer monitoring in a model filamentous fungus host, *Cryphonectria parasitica*

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ABSTRACT

The ascomycete *Cryphonectria parasitica* has served as a model filamentous fungus for studying virus host interactions because of its susceptibility to diverse viruses, its genetic manipulability and the availability of many biological and molecular tools. *Cryphonectria prasitica* is known to activate antiviral RNA silencing upon infection by some viruses via transcriptional up-regulation of key RNA silencing genes. Here, utilizing a newly developed GFP-based reporter system to monitor dicer-like 2 (*dcl2*) transcript levels, we show different levels of antiviral RNA silencing activation by different viruses. Some viruses such as mycoreovirus 1, a suppressor-lacking mutant of Cryphonectria hypovirus 1 (CHV1-Δp69) and Rosellinia necatrix partitivirus 11 (RnPV11) highly induced RNA silencing, while others such as CHV3, Rosellinia necatrix victorivirus 1 and RnPV19 did not. There was considerable variation in *dcl2* induction by different members within the family *Hypoviridae* with positive-sense single-stranded RNA genomes or *Partitiviridae* with double-stranded RNA genomes. Northern blotting and an *in vitro* Dicer assay developed recently by us using mycelial homogenates validated the reporter assay results for several representative virus strains. Taken together, this study represents a development in the monitoring of Dicer activity in virus-infected *C. parasitica*.

## 1. Introduction

RNA silencing is a primary antiviral host defense that is conserved across eukaryotes (Aliyari et al., 2008; Berkhout, 2018; Deleris et al., 2006; Du et al., 2011; Pumplin & Voinnet, 2013) in which virally derived dsRNAs are cleaved by Dicer into small dsRNAs; one of the strands is then incorporated into the Argonaute (Ago) effector complex and guide the effector to target homologous RNA molecules for degradation by its RNase H-like activities. *Cryphonectria parasitica*, a filamentous ascomycete causing destructive chestnut blight, has been studied from the biological control viewpoints because *C. parasitica*-infecting hypoviruses confer hypovirulence to the fungus (Nuss, 2005). The fungus also serves as a model host for studying virus/host and virus/virus interactions (Eusebio-Cope et al., 2015). Importantly, the fungus can support many viruses including homologous viruses isolated from this fungus and heterologous viruses isolated from different fungal species even belonging to different subclasses. Such viruses include diverse positive-sense (+), single-stranded (ss) RNA viruses and double-stranded (ds) RNA viruses that are capsidless or encapsidated and are replicated in different subcellular sites. The *C. parasitica*/viruses provides a unique platform for exploring antiviral RNA silencing where a relatively smaller number of players, i.e. dicer-like 2 (*dcl2*) and argonaute-like 2 (*agl2*), play key roles (Nuss, 2011; Segers et al., 2007; Sun et al., 2009) and are transcriptionally induced upon virus infection (Andika et al., 2017; Chiba & Suzuki, 2015; Sun et al., 2009; Zhang et al., 2012).

The transcriptional upregulation of *dcl2* and *agl2* upon infection by some viruses reaches to ~40-fold relative to virus free fungi and requires the SAGA (*S*pt-*A*da-*G*cn5 acetyltransferase) complex, a well-known transcriptional coactivator. This is the case for infection by a mutant of the prototype hypovirus Cryphonectria hypovirus 1 (CHV1) that lacks the RNA silencing suppressor (RSS) p29 (Chiba & Suzuki, 2015; Sun et al., 2009). Thus, p29 is considered to cancel the upregulation of the RNA silencing-related genes. Some other viruses include a reovirus, mycoreovirus 1 (MyRV1) and a partitivirus, *Rosellinia necatrix*

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partitivirus 3 (RnPV3). These viruses activate an antiviral RNA silencing state highly enough to eliminate a pre-existing victorivirus (Chiba et al., unpublished results). Furthermore, Aulia et al. has shown that the stable infection by another mycoreovirus, MyRV2, requires inhibition of RNA silencing by a hypovirus, CHV4 (Aulia et al., 2019). On the other hand, others viruses such as the victorivirus Rosellinia necatrix victorivirus 1 (RnVV1) and Cryphonectria mitovirus 1 (CpMV1) do not apparently induce *dcl2* and *agl2* (Chiba & Suzuki, 2015; Shahi et al., 2019). It is of interest to note that DCL2 is likely required as a positive feedback player in SAGA-mediated transcriptional induction. It is also noteworthy that a number of other host genes are induced by this pathway upon virus infection, and importantly some are involved in the mitigation of virus symptom induction (Andika et al., 2019). It remains elusive as to how host fungi perceive virus infections and induce antiviral RNA silencing and how particular viruses avoid this host RNA silencing induction.

In this study, we developed a GFP-based reporter system to monitor antiviral RNA silencing induction using the *dcl2* promoter. This reporter system showed high-level transcriptional induction of antiviral RNA by some RNA viruses that have been unexplored in this respect. Furthermore, the activated RNA silencing levels were validated by northern blotting of *dcl2* transcripts and by an *in vitro* Dicer assay for several representative virus-infected fungal strains.

## 2. Materials and Methods

### 2.1. Fungal and viral materials

The GFP reporter strain termed EP155-*dcl2pro*::*egfp*, with the genetic background of the standard strain EP155, was previously described by Andika et al. (Andika et al., 2017), in which a GFP coding domain was fused with the *C. parasitica dcl2* promoter region of approximately 2 kbp. We also previously prepared EP155 infectants with various different viruses (Eusebio-Cope et al., 2015; Telengech et al., 2020). To transfer the viruses tested in this study (Table 1), EP155 singly infected with viruses were cocultured with the reporter strain and virus lateral transfer was confirmed by phenotypic observation and RT-PCR analysis. These fungal strains were grown on Difco$^{TM}$ potato dextrose agar (PDA) plates for phenotypic observation and PDA-cellophane for nucleic acid extraction and reporter assays (Andika et al., 2017).

### 2.2. RNA extraction and northern analyses

Total RNA fractions were prepared from PDF-cellophane cultures as described earlier (Eusebio-Cope & Suzuki, 2015). Single-stranded RNA fractions were enriched by $LiCl_2$ precipitation. After normalizing against rRNA, equal amounts of RNA were electrophoresed in agarose gel under denaturing conditions and blotted onto nylon membrane. Dioxigenin (DIG)-11-dUTP-labeled PCR fragments of the GFP coding domain and *dcl2* were prepared by the method recommended by the manufacturer (Roche Diagnostic, Mannheim) and used as probes. Primers' sequences have been described earlier by Andika et al. (Andika et al., 2017).

### 2.3. GFP reporter assay

For preparation of fungal culture used for observation of GFP expression, a micro cover glass (22 mm × 22 mm; thickness, 12–17 mm) (Matsunami, Japan) were placed on top of cellophane PDA, and, on the edge of micro cover glass, a small plug of fungus was cultured for 3 days. The mycelia grew through the micro cover glass after several days. This micro clover glass was detached and placed on a micro slide glass (Matsunami). GFP expression was observed using an Olympus Fluoview FV1000 confocal laser scanning, microscope (Olympus, Japan).

### 2.4. In vitro assay for dicer activity

Fungal colonies were grown in potato dextrose broth (PDB) medium (Becton, Dickinson and Co., NJ, USA) at 22 °C for 5 days, then homogenized in 6 mL $g^{-1}$ of extraction buffer containing 20 mM TrisHCl (pH 7.5), 4 mM $MgCl_2$, 5 mM DTT, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 μg $mL^{-1}$ leupeptin, and 1 μg $mL^{-1}$ pepstatin A using liquid nitrogen after air-drying on the benchtop. Samples were then centrifuged at 15,000 rpm at 4 °C in a microcentrifuge, then homogenates were used in the *in vitro* Dicer assay as reported for plant materials (Nagano et al., 2014; Tabara et al., 2018). Complementary single-stranded 50-nt RNAs were synthesized and labeled at the 5′-terminus using T4 polynucleotide kinase (Takara, Japan) and [γ-$^{32}$P]-ATP. After annealing, the resulting end-labeled dsRNA was used as a substrate. Digested products were analyzed by polyacrylamide gel electrophoresis under urea-denaturing conditions. The relative band intensities of the small RNA products were quantified by a Typhoon FLA 7000 image analyzer (GE Healthcare,

**Table 1**
Virus strains tested in this study.

| Family | Virus strain | Strain abbreviation | Accession | Reference |
|---|---|---|---|---|
| *Hypoviridae* | Cryphonectria hypovirus 1- EP713 | CHV1 (wild-type) | M57938 | Shapira et al. (1991) |
| | | CHV1-Δp69 (ORF A deletion mutant) | | Suzuki and Nuss (2002) |
| | Cryphonectria hypovirus 2-NB58 | CHV2 | L29010 | Hillman et al. (1994) |
| | Cryphonectria hypovirus 3- GH2 | CHV3 | AF188515 | Smart et al. (1999) |
| *Narnaviridae* | Cryphonectria mitovirus 1-NB631 | CpMV1 | L31849 | Polashock and Hillman (1994) |
| *Reoviridae* | mycoreovirus 1-9B21 | MyRV1 | AY277888 ~ AY277890<br>AB179636 ~ AB179643 | Suzuki et al. (2004) |
| *Totiviridae* | Rosellinia necatrix victorivirus 1-W1032 | RnVV1 | AB742454 | Chiba et al. (2013) |
| *Megabirnaviridae* | Rosellinia necatrix megabirnavirus 1-W779 | RnMBV1 | AB512282<br>AB512283 | Salaipeth et al. (2014) |
| *Partitiviridae* | Rosellinia necatrix partitivirus 3-W118 | RnPV3 | LC010950<br>LC010951 | Telengech et al. (2020) |
| | Rosellinia necatrix partitivirus 6-W558 | RnPV6 | LC010952<br>LC010953 | Telengech et al. (2020) |
| | Rosellinia necatrix partitivirus 11-W98 | RnPV11 | LC517370<br>LC517371 | Telengech et al. (2020) |
| | Rosellinia necatrix partitivirus 14-W744 | RnPV14 | LC517376<br>LC517377 | Telengech et al. (2020) |
| | Rosellinia necatrix partitivirus 18-W442 | RnPV18 | LC517384<br>LC517385 | Telengech et al. (2020) |
| | Rosellinia necatrix partitivirus 19-W442 | RnPV19 | LC517386<br>LC517387 | Telengech et al. (2020) |
| | Rosellinia necatrix partitivirus 20-W1134 | RnPV20 | LC517388<br>LC517389 | Telengech et al. (2020) |

IL, USA). The small RNA products were calculated as the relative band intensity of the 18–25 nt RNAs in comparison with the total intensity of all bands in each lane. Data were compared by analysis of variance followed by a Dunnett's test to compare to virus free EP155.

## 3. Results

### 3.1. Development of a GFP reporter assay system for monitoring antiviral RNA silencing

We had already identified the promoter region of *dcl2* responsible for transcriptional induction via MyRV1 and CHV1-Δp69 (Andika et al., 2017). The 2-kbp promoter region was fused with the eGFP coding domain within the expression vector pCPXHY3. Transformants with this construct were obtained and tested for GFP fluorescence before and after infection by MyRV1 and CHV1-Δp69, which are known for their high degree of *dcl2* transcriptional inducibility. Transformants fluoresced in green only upon infection by either virus (Fig. 1A). However, no green fluorescence was observed when the reporter fungal strain was infected with the wild-type strain (WT) CHV1 or uninfected (Fig. 1A).

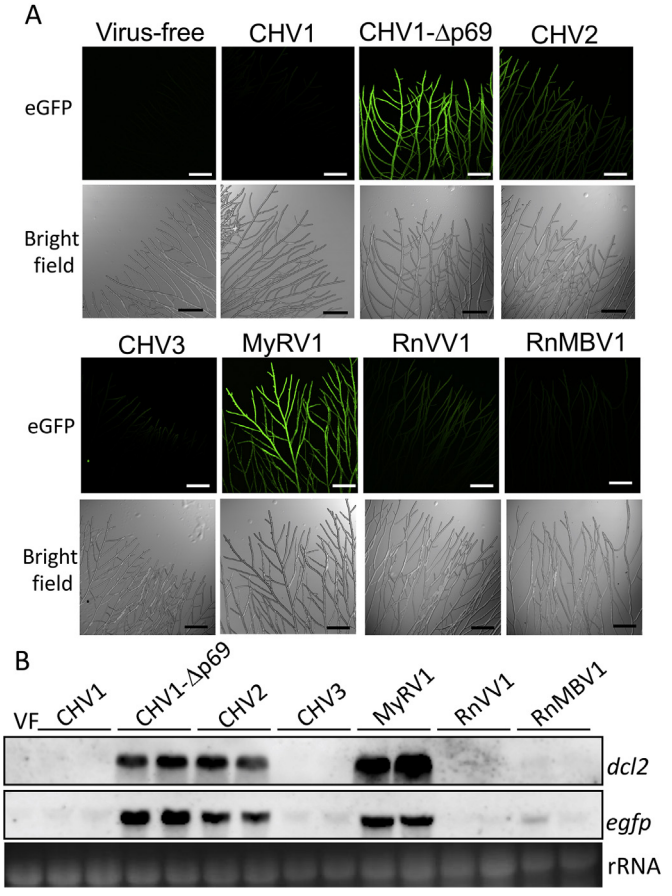


**Fig. 1. Assessment of Dicer-mediated antiviral defense upon virus infection.** GFP fluorescence exhibited by the reported strain infected by different viruses. The *C. parasitica* reporter strain, carrying an *egfp* gene under the control of the *dcl2* promoter, was infected singly by diverse groups of viruses indicated: wt CHV1, an RSS-lacking CHV1 mutant Δp69, CHV2, CHV3, MyRV1, RnVV1 or RnMBV1 (see Table 1). The virus-free reporter strain was analyzed in parallel. These fungal strains were subjected to confocal laser scanning microscopy under the same conditions. White and black bars in this and Fig. 3A indicate 100 μm. (B) Northern blotting of virus-infected reporter strains. Total single-stranded RNA fractions were obtained from two biological replicates of the virus-infected reporter strains shown in (A). Transcripts of *dcl2* and *egfp* were detected by the respective DNA probes (see Materials and Methods). rRNA (28S) was used as a loading control.

### 3.2. Comparison of GFP-green fluorescence levels among the reporter strains infected with different viruses

We first tested a total of seven different dsRNA and ss (+)viruses, including the aforementioned viral strains, that were previously known and unknown about their ability to induce the *dcl2* gene. Several well-characterized viruses were selected whose ability to induce *dcl2* was previously known, including CHV1, CHV1-Δp69, MyRV1 and RnVV1, while CHV2, CHV3 and RnMBV1 had not been unexplored in this respect. As shown in Fig. 1A, in contrast to the reporter strains infected by CHV1-Δp69 and MyRV1, those infected by CHV1-wt, CHV3, RnVV1 and RnMBV1 showed no or little fluorescence. CHV2 induced an intermediate level of green fluorescence. Also included in this assay was a mitochondrially replicating (+)RNA virus, CpMV1, that has been shown to be unable to induce silencing genes by Shahi et al. (Shahi et al., 2020). As expected from its inability to transcriptionally upregulate *dcl2*, no green fluorescence was observed in CpMV1-infected reporter strains (data not shown).

Taken together, the GFP reporter assay using a confocal laser scanning microscopy showed variations in fluorescence levels depending on which virus infected the fungal reporter strain.

### 3.3. Validation of GFP-green fluorescence levels by northern blotting of dcl2 and GFP reporter mRNAs

Fig. 1A shows the considerable variability of dcl2 transcriptional inducibility by different viruses; for example, a CHV1 mutant lacking the p29 RNA RSS (CHV1-Δp69) upregulates *dcl2*, while wt CHV1 does not. To validate the reporter assay results, we conducted northern analyses of *egfp* and *dcl2* mRNAs (Fig. 1B). The highest levels of *dcl2* was detectable in MyRV1-infected fungal strains, while relatively lower levels of *dcl2* transcripts were detected in fungal reporter strains infected by CHV1-Δp69 and CHV2 compared to the former strains. *egfp* mRNA accumulated to in CHV1-Δp69- and MyRV1-infected strains at the highest level in all tested strains, while CHV2 induced a slightly lower level of *egfp* mRNA. No or few transcripts of *dcl2* or *egfp* were detectable in fungal reporter strains uninfected (VF) or infected by wt CHV1, CHV3, RnVV1 or RnMBV1 (Fig. 1B). Importantly, a very similar induction pattern was observed for *dcl2* mRNA and the reporter eGFP mRNA, which was driven by the *dcl2* promoter.

### 3.4. Detection of dicer activities in mycelial homogenates

We recently have developed a biochemical method for detecting Dicer enzymatic activities in homogenates from various plant organs (Nagano et al., 2014; Tabara et al., 2018). We applied it to *C. parasitica*. First, we compared Dicer activities between three genetic backgrounds: wild-type, Δ*dcl1* and Δ*dcl2*. As a result, WT EP155 and Δ*dcl1* yielded 21-nt and 23-nt small RNAs, possible cleavage products of 50-nt small RNA substrates added (see Materials and Methods). By contrast, no such Dicer products were detected in homogenates from Δ*dcl2* or a negative control (substrate without mycelial homogenates). This is consistent with the observation made by Nuss and colleagues that *dcl2* is responsible for anti-viral RNA silencing (Segers et al., 2007).

A few representative virus strains showing different *dcl2* and eGFP induction patterns (Fig. 1) such as MyRV1 CHV1-Δp69, CHV2 and CHV3 were selected for this enzymatic assay. Greater amounts of digested small RNAs were detected in fungal strains infected by CHV1-Δp69 and MyRV1, while slightly lower amounts of small RNAs, relative to the former two strains, were observed in CHV2-infected fungal strain (Fig. 2). CHV3-infected mycelial extracts led to the digestion at a level similar to that from the virus free strain. These results are generally consistent with the results of the GFP reporter assay (Fig. 1).

At least two size classes (21 and 23 nt) of small RNAs were detected *in vitro* Dicer assay (Fig. 2). Previous studies showed a peak at 21-nt virus-derived small RNAs in *C. parasitica* (Andika et al., 2017; Zhang et al., 2008). Similar *in vitro* studies with *Brassicaceae*, rice and maize tissue

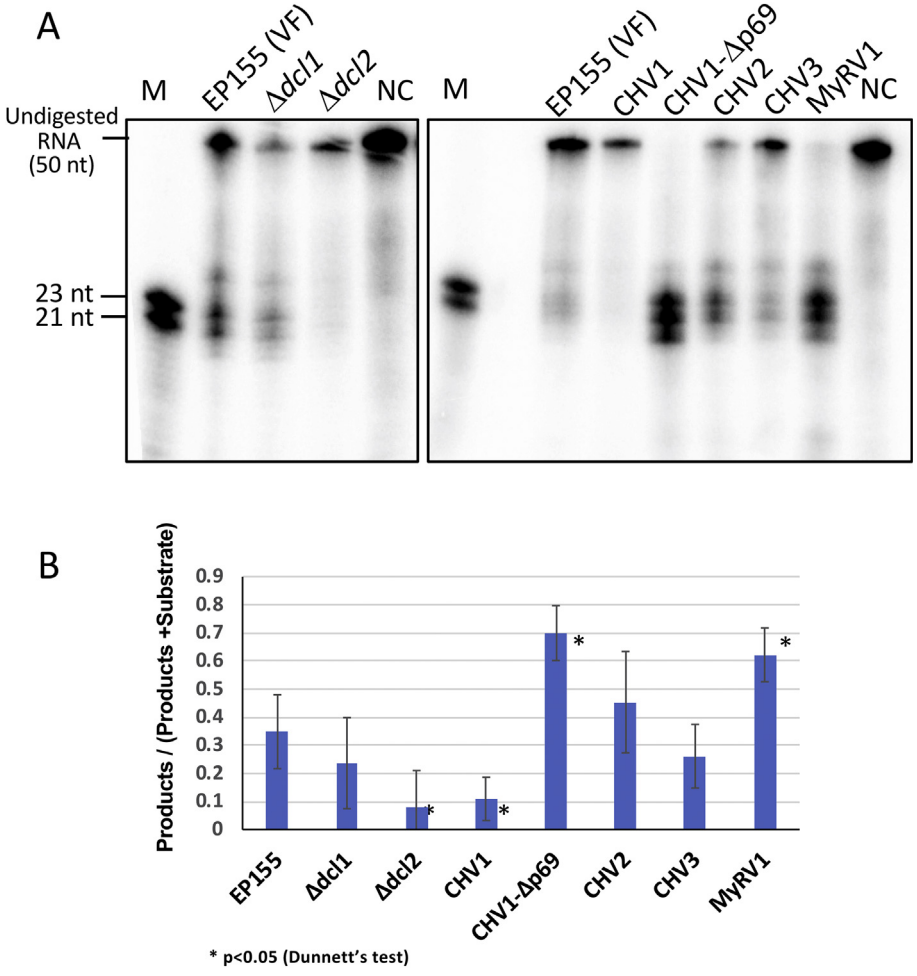


**Fig. 2.** ***In vitro*** **Dicer activities detected in mycelial extracts. (A)** Dicer activities in mycelial homogenates derived from fungal strains infected by different viruses shown on the top of the autoradiograph. Synthetic $^{32}$P-labeled dsRNA of 50 bp with 2-nt 3′overhangs was used a substrate and cleaved products by Dicer was analyzed by polyacrylamide gel electrophoresis and autoradiography. The cleavage products of 21 and 23 nt are indicated by arrows. Enzymatic activities were quantified as described by (Nagano et al., 2014). Lane M refers to size markers of small RNAs of 23 and 21 nt. **(B)** Quantification of dicing activities. The relative band intensity of DCL2 products were calculated as described in Materials and Methods using a Typhoon FLA image analyzer (GE Healthcare). Means with standard deviations were calculated from three replicates.

extracts showed the same size classes of small RNAs as those detected *in vivo* (Nagano et al., 2014; Tabara et al., 2018). Whether 23-nt small RNAs detected *in vitro* reflects *in vivo* Dicer activity in *C. parasitica* remains unknown.

Note that lane NC (negative control) without mycelial homogenate showed greater substrate band intensity than some other lanes such as CHV1 and Δ*dcl2* (Fig. 2A). This may be explained by the presence of unidentified nucleases in mycelial homogenates of fungal origin.

### 3.5. Different degrees of dcl2 induction by different partitiviruses

Partitiviruses are omnipresent in fungi and plants. We previously transfected EP155 with diverse partitiviruses from another phytopathogenic fungus, *R. necatrix* (Telengech et al., 2020) and horizontally transferred into the reporter strain via anastomosis. Utilizing the validated GFP reporter assay, we examined seven characterized partitiviruses from *R. necatrix*: RnPV3, RnPV6, RnPV11, RnPV14, RnPV18, RnPV19, and RnPV20, for their ability to induce *dcl2* transcription. These viruses, which belong to the genus *Betapartitivirus* except for the alphapartitivirus RnPV19, were singly introduced into the GFP reporter strain (Table 1). As shown in Fig. 3A, different levels of green fluorescence were observed in reporter strains infected by different partitiviruses. For example, the highest levels of green fluorescence were recorded in fungal strains infected with RnPV11, RnPV18, and RnPV20 (Fig. 3A), as in the case for CHV1-Δp69 (data not shown), whereas no or little green fluorescence was detectable in the virus-free strains or strains infected with RnPV3 or RnPV19. RnPV6- and RnPV14-infected fungal strains showed intermediate green fluorescence.

The results of northern blotting (Fig. 3B) were congruent with those of the GFP reporter assay (Fig. 3A). Namely, fungal strains infected by RnPV11 and RnPV20 accumulated *dcl2* and *egfp* mRNA most abundantly, almost the same level as that in CHV1-Δp69 infected strain, whereas no or few of those were detected in the fungal strains infected by RnPV3 or RnPV19. A slightly inconsistent result was that the RnPV18-infected fungal strain showed a *dcl2* level similar to the RnPV6-infected strain, which was lower than that expected based on the green fluorescence level.

Taken together these results indicate that partitiviruses vary in their induction of antiviral RNA silencing.

## 4. Discussion

It has recently been revealed the Dicer plays important dual functional roles of Dicer in the multilayer antiviral defense in *C. parasitica*: one in antiviral RNA silencing and the other in SAGA-mediated transcriptional upregulation of many virus-responsive host genes, which include *dcl2* and *agl2*, leading to the mitigation of symptoms (Andika et al., 2017, 2019; Segers et al., 2007). For the first function, the induction level appears to represent the antiviral RNA silencing level, given the observation that a susceptible virus can no longer replicate under the highly *dcl2*-induced state. RNA silencing-related genes were also reported to be transcriptionally induced in other insect, plant and fungal fungal hosts (Spellberg & Marr, 2015; Xie et al., 2001; Xu et al., 2012). In fungi, the transcriptional upregulation of RNA silencing-related genes is suppressed by RNA silencing suppressors from viruses such as CHV1 and possibly CHV4 (Aulia et al., 2019; Chiba & Suzuki, 2015; Sun et al.,

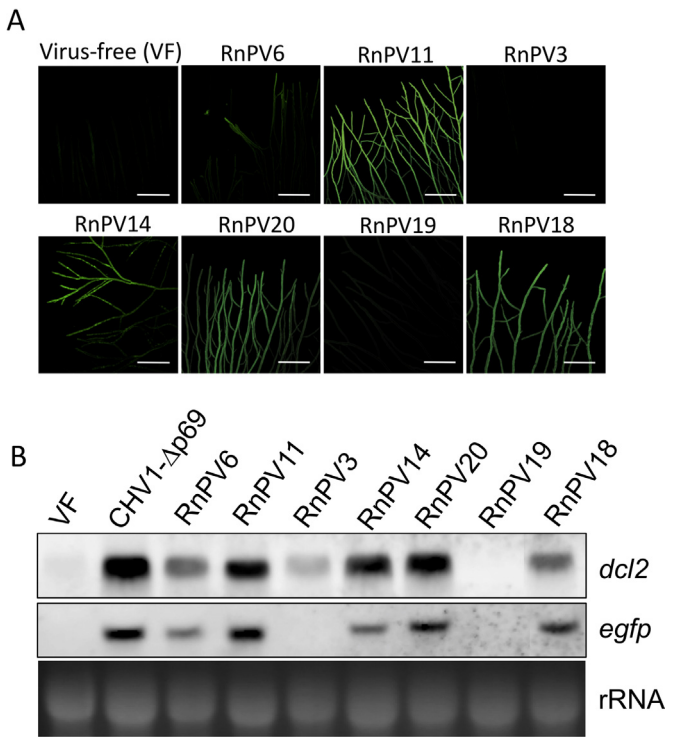


**Fig. 3. GFP reporter assay of *dcl2* induction by different partitiviruses. (A)** GFP fluorescence detected in mycelia infected by diverse partitiviruses. The reporter fungal strains singly infected by RnPV3, RnPV6, RnPV11, RnPV14, RnPV18, RnPV19 and RnPV20 were subjected to confocal microscopy as described in the Materials and Methods. **(B)** Northern blotting of virus-infected reported strains. The same set of fungal strains as in (A) were analyzed by northern blotting. Note that all of these partitiviruses were from a heterologous ascomycete, *R. necatrix*. Virus-free and CHV1 Δp69-infected reported strains were tested in parallel.

2009). However, a moderate constitutional level of *dcl2* transcription under the control of the heterologous cryparin gene promoter is sufficient for the second role (Andika et al., 2019). Therefore, monitoring *dcl2* expression upon virus infection in *C. parasitica* is a very important step toward understanding host defense/virus counter-defense responses.

This study developed a fluorescent protein-based reporter assay to monitor the activation of antiviral RNA silencing via transcriptional upregulation of *dcl2* upon virus infection (Fig. 1). The method was validated by two other methods: northern blotting and an *in vitro* enzymatic assay of DCL2 (Figs. 1 and 2). The fungus *C. parasitica*, perceives some viruses, not all, by a host unknown sensor system, and transcriptionally induces antiviral defense genes such as *agl2* and *dcl2* (Sun et al., 2009; Zhang et al., 2008), and other host genes (Andika et al., 2019). The upregulation of *agl2* and *dcl2* requires a general transcriptional co-factor, the SAGA complex and DCL2. Whether AGL2 is needed for the induction of *dcl2* depends on the virus (Chiba & Suzuki, 2015). Another interesting phenomenon with antiviral RNA silencing activation is the variation in the induction levels manifested by different viruses, even by different strains belonging to the same species (Chiba et al., 2016; Chiba & Suzuki, 2015; Shahi et al., 2019; Zhang et al., 2012). The reporter assay allowed for the estimation of antiviral RNA silencing activation by diverse viruses as listed in Table 1. The tested viruses include (+)RNA viruses such as CHV1 and its mutant CHV1-Δp69, CHV2, and CHV3, and dsRNA viruses including partitviruses, a mycoreovirus, and a victorivirus. The reporter assay categorized these viruses into three groups: one induced *dcl2* to high levels, another did not induce *dcl2,* and one group was intermediate. It is unknown what governs the transcriptional *dcl2* upregulation levels. It seems that once the host fungus senses a virus infection, it induces *dcl2* full-scale in most cases.

We recently revealed that a same subset of host genes including RNA silencing-related genes are transcriptionally upregulated by virus infection and transgenic dsRNA expression (Choudhary et al., 2007; Honda et al., 2020). It should be noted that, in *Neurospora crassa*, a reporter system for the dsRNA response has been established (Li et al., 2011). However, it remains elusive as to how host fungi perceive RNA viruses or dsRNA production and activate antiviral RNA silencing via transcriptional upregulation. How much virus accumulates is possibly an important factor for triggering this response. However, the *dcl2* induction level may not be determined by the virus accumulation level, but rather is determined by complex factors. This can be seen in the prototype hypovirus CHV1-EP713. CHV1-Δp69 accumulates 10-fold less compared to the wild-type CHV1 (Suzuki & Nuss, 2002), largely because of the lack of an RSS (Sun et al., 2009; Chiba & Suzuki, 2015). Of note is that different viruses originally from the white root fungus, belonging to the genera *Alpha-* or *Betapartitivirus*, showed different levels of *dcl2* induction (Fig. 3). These partitiviruses were characterized biologically and molecularly (Telengech et al., 2020). GFP inducibility does not necessarily correlate with symptom inducibility or virus accumulation levels. Although not reported from partitiviruses, partitiviruses RSSs may be different in RNA silencing suppression levels.

The *in vitro* assay for Dicer activity is well-established for some eukaryotes such as *Drosophila* embryo lysates (Haley et al., 2003; Tuschl et al., 1999; Zamore et al., 2000). Organisms with a cell wall, such as plants and fungi, are generally difficult to work with for biochemical and enzymatic assays. However, Fukuhara and colleagues established a protocol for examining Dicer activity in the model plant, *Arabidopsis thaliana* using *in vitro* synthesized [$\gamma$-$^{32}$P]-UTP-labeled dsRNAs (Nagano et al., 2014; Tabara et al., 2018). An *in vitro* Dicer assay was also developed for the model filamentous fungus, *N. crassa* by Cogoni and colleagues (Catalanotto et al., 2004), and later its enzymatic characteristics were revealed by Fukuhara's group (unpublished results). This study adopted the protocol of preparing mycelial homogenates of *C. parasitica*. As mentioned above, the method is useful to determine which viruses induce the antiviral defense in *C. parasitica* (this study). Annisa et al. (Aulia et al., 2019) identified viruses with RSS capability by coinfecting a reporter strain pre-infected by a highly *dcl2*-inducing virus with the candidate virus. Furthermore, this study provided interesting insights into enzymatic properties of Dicer. Nuss and colleagues previously showed that while *C. parasitica* has two *dcl* genes, *dcl1* and *dcl2*, only *dcl2* plays a role in antiviral RNA silencing and is responsible for virus-derived small RNA production (Segers et al., 2007; Sun et al., 2009; Zhang et al., 2008). The developed protocol failed to detect Dicer activities in Δ*dcl2*. This observation confirmed the previous results and suggests that DCL1 shows no or little cleavage activity for the dsRNA used under the assay conditions regardless of whether the host is infected or uninfected by virus.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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